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OSMOREGULATION IN A MARINE CILIATE, *MIAMIENSIS AVIDUS*. I. REGULATION OF INORGANIC IONS AND WATER[1]

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Little is known of the content and regulation of inorganic ions in marine protozoa. The ionic composition of *Uronema filificum* (Thompson and Kaneshiro, 1968), a marine ciliate, has been determined (Kehlenbeck *et al.*, 1965) and certain aspects of contractile vacuole function have been studied in several forms (Kitching, 1938, 1967).

Miamiensis avidus (Thompson and Moewus, 1964), a euryhaline ciliate, can be studied after exposure to large changes in environmental osmolarity. Also, it grows axenically in mass cultures and has a short generation time, so chemical analyses are feasible. Taking advantage of these features, we have sought information on the function of the contractile vacuole and on the kinds and concentrations of internal inorganic ions.

Materials and Methods

Miamiensis avidus, a facultative parasite of seahorses was obtained from Dr. Liselotte Moewus and maintained axenically in a medium of the following composition: lactalbumin hydrolysate solution (10%, w/v) (Nutritional Biochemicals Corp.) 10 ml, calf serum (Grand Island Biological Co.) 5 ml, filtered sea water 85 ml. All components were sterilized separately and combined aseptically. The complete medium was adjusted to pH 7.5 with sterile 4.2% $NaHCO_3$ (Moewus, 1963).

Intracellular inorganic ion analyses

One-liter mass cultures were grown in 2500-ml low-form Erlenmeyer flasks at 24–26° C. A 20-ml inoculum of a log phase culture was introduced into each flask. Cells in late log phase to early stationary phase were concentrated by centrifugation in 40-ml conical tubes at 300 *g* for 3 min and then exposed to various experimental conditions.

Appropriate concentrations of artificial sea water (100% = 31‰), a solution composed of the major salts found in sea water (Marine Biological Laboratory Formulae and Methods V., 1964), were added to cell suspensions to produce high osmolarity test environments. Distilled water was added for low osmolarity envi-

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ronments. Sucrose (0.72 *M*) isosmotic to M.B.L. sea water was used in some experiments to maintain osmotic pressure when inorganic ion concentrations were being varied. In ion substitution experiments, Na^+, K^+, and Ca^{++} were replaced by choline$^+$. Mg^{++} was replaced by Na^+, and Cl^- was replaced by proprionate$^-$ and SO_4^{--}.

C^{14}-inulin was added to cell suspensions within 2 min prior to centrifugation (1000 *g* for 5 min) for estimation of extracellular space (Dunham and Child, 1961). An extracellular space of 25%, based on a mean of 106 determinations, was used in all calculations of cellular ion content. Duplicate 0.1-ml aliquots of cell suspensions were preserved for counting cell numbers in a Sedgewick-Rafter chamber with the aid of a Whipple ocular micrometer. Cell volume was calculated using the equation:

$$\text{volume/cell} = \frac{(\text{wet wt of pellet})\ (1\text{-inulin space})}{\text{no. cells/10 ml cell suspension}}$$

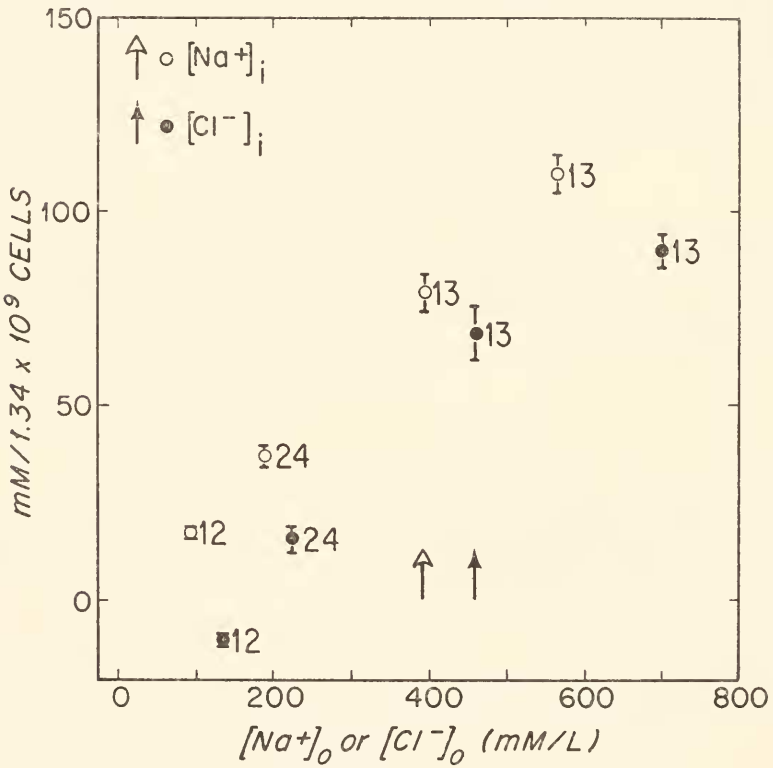

FIGURE 1. Changes in $[Na^+]_i$ and $[Cl^-]_i$ of *M. avidus* (expressed as m*M*/initial liter of cells) following changes in $[Na^+]_o$ and $[Cl^-]_o$. In this and the following figures, ion concentrations are expressed in m*M*/original liter of cells, *i.e.*, expressed in terms of the number of cells in the initial control liter of cells (kg cells), since the volumes of cells change under various experimental conditions. Means, standard errors of the means, and number of determinations are indicated. Arrows show values in 100% sea water.

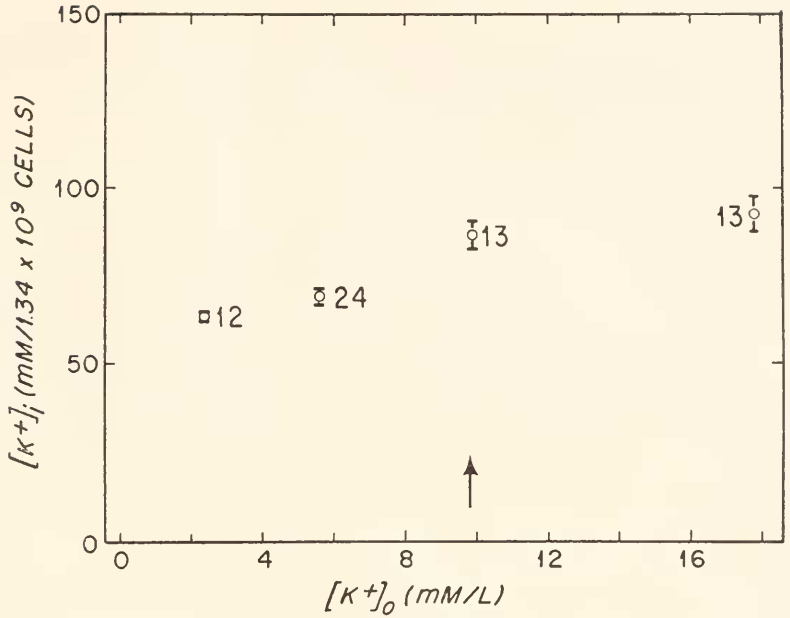

FIGURE 2. Changes in $[K^+]_i$ of *M. avidus* following changes in $[K^+]_o$.

Inorganic ions were extracted in 10 ml of dilute acetic acid. The extraction tubes were placed in a 100° C water bath and allowed to stand for at least an hour. After extraction, the debris was packed by centrifugation and the supernatant decanted. Values obtained for the ion contents of ciliates were corrected for extracellular space and cell volume changes.

Na^+, K^+, Ca^{++} and Mg^{++} were determined with a Coleman photometer and Cl^- by electrometric titration (Aminco-Cotlove titrator).

Contractile vacuole output

Frequency of vacuole pulsation in different solutions was observed directly with a phase contrast microscope.

To compare the vacuole output in different test solutions, ciliates were equilibrated for more than 30 min and were observed and photographed within 5 hours. Time-lapse photographs (Sage Cinephotomicrographic Apparatus, Model 500, Bolex H16 M camera) were taken at 60 frames/min and 420 × magnification. Nothing was done to immobilize the ciliates, and those that showed signs of compression by the cover slip (vesiculated cytoplasm or everted oral area) were not included in the measurements. A stage micrometer was filmed for calibration purposes. Cell length and width, and contractile vacuole diameter were measured with the aid of an analytical movie projector. For calculation of cell volume the shape of the vacuole was considered to be a sphere, and the shape of the ciliate to be a cone plus a hemisphere. Values for cell volume obtained by this method agreed with those described above.

Vacuolar rate was expressed as volume output/unit time. Volume output/unit time was divided by the cell volume to give the fraction of cell volume turnover/unit time. Visual observations and photography were completed within 5 min after slides of equilibrated ciliates were prepared for examination.

Results

Intracellular inorganic ion concentration in Miamiensis avidus

When the intracellular concentrations of inorganic ions were determined in ciliates from 100% sea water, and from various concentrations above and below 100% 30 min after a change in the external salinity, it was found that intracellular Na^+ ($[Na^+]_i$) and Cl^- ($[Cl^-]_i$) were lower than environmental values ($[Na^+]_o$, $[Cl^-]_o$) [5] (Fig. 1). They increased as external salinity was increased and decreased as external salinity was decreased, but always remained lower than the environ-

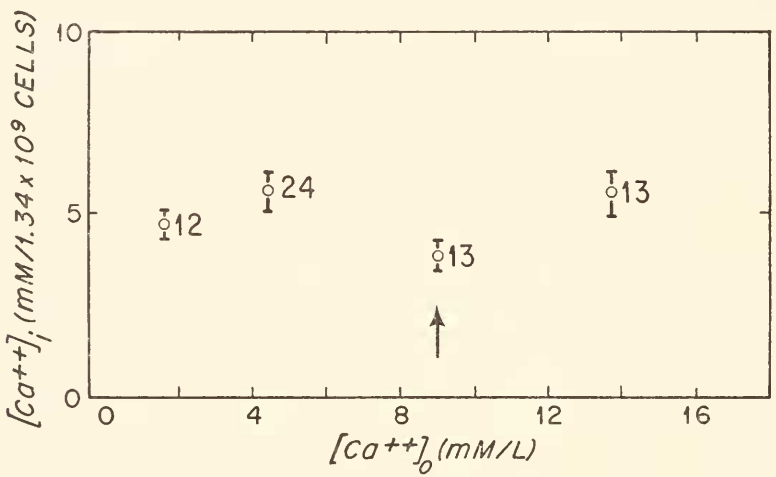

Figure 3. Changes in $[Ca^{++}]_i$ of *M. avidus* following changes in $[Ca^{++}]_o$.

ment. The ciliates maintained $[Na^+]_i$ at 1/5, and $[Cl^-]_i$ at about 1/7 the environmental values at all sea water concentrations tested. Since $[Cl^-]_i$ is very low relative to $[Cl^-]_o$, estimates of $[Cl^-]_i$ are particularly subject to large variability with slight errors in determinations of extracellular space. The negative value for $[Cl^-]_i$ in 25% sea water might be accounted for in these terms.

$[K^+]_i$ was considerably higher than $[K^+]_o$ in the ciliates from 100% sea water and remained at a fairly constant concentration over the range of external salinity changes tested (Fig. 2). Atypical of most cells, ciliates in 100% and 150% sea water had $[Na^+]_i$ greater than $[K^+]_i$ since $[Na^+]_i$, but not $[K^+]_i$, increased as external salinity was increased. $[K^+]_i$ was greater than $[Na^+]_i$ in ciliates in 50% and 25% sea water.

[5] [ ]$_i$—intracellular concentration (m*M*/original liter cells); one liter cells was taken to be equivalent to one kg cells.
[ ]$_o$—extracellular concentration (m*M*/liter).

TABLE I

Intracellular inorganic ion concentrations of M. avidus cultured and equilibrated in sea water-culture media

% Sea water-culture medium (exposure time)	mM/kg cells* [±1.4 – 2.7 mM (SE)]			
	$[Na^+]_i$	$[K^+]_i$	$[Cl^-]_i$	N**
100% (2 years)	87.9	73.7	60.8	104
50% (3 months)	52.8	60.6	24.9	31
100% (2 years) 50% (30 min)	37.1	69.8	16.0	24

*mM/1.44 × 10^9 cells (1 liter).
** No. determinations.

$[Ca^{++}]_i$ and $[Mg^{++}]_i$ were lower than $[Ca^{++}]_o$ and $[Mg^{++}]_o$ in ciliates from 100% sea water and remained constant despite changes in salinity (Figs. 3, 4). Internal concentrations of these ions were, therefore, higher than the external concentrations when the ciliates were in diluted sea water and lower than external concentrations when they were in 150% sea water.

When the external environment was diluted by one-half (to 50% sea water) or made more concentrated (to 150% sea water), and the inorganic ion content of the ciliates analyzed at different times thereafter, the net movements of monovalent ions (Na^+, Cl^- and K^+; Figs. 5, 6, 7) were essentially completed about 10 min after exposure to the new conditions. There were no net movements of Ca^{++} and Mg^{++}.

Ion concentrations of ciliates subcultured for 2 years in 100% sea water-culture medium were also compared with the following: ciliates subcultured for 3 months

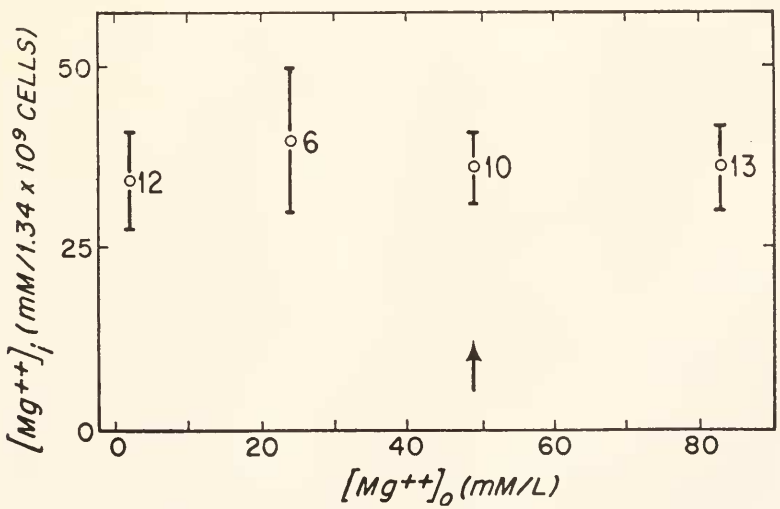

FIGURE 4. Changes in $[Mg^{++}]_i$ of *M. avidus* following changes in $[Mg^{++}]_o$.

TABLE II

Intracellular inorganic ion concentrations of cultured U. filificum

% Sea water-culture medium	mM/kg cells		
	[±2.3 – 9.4 mM (SE) in 22 determinations each ion]		
	$[Na^+]_i$	$[K^+]_i$	$[Cl^-]_i$
100%	84.4	134.1	16.1
50%	63.8	102.9	1.7

in 50% sea water-culture medium and then equilibrated in 50% sea water-culture medium for 30 min (Table I). $[Na^+]_i$ and $[Cl^-]_i$ were lower in ciliates subcultured in 50% sea water-culture medium than in ciliates subcultured in 100% sea water-culture medium. They were higher in ciliates subcultured in 50% sea water-culture medium than in ciliates subcultured in 100% sea water-culture medium and then equilibrated in 50% sea water-culture medium for 30 min. $[K^+]_i$ and $[Ca^{++}]_i$ were about the same under all three conditions. $[Mg^{++}]_i$ was not determined.

Intracellular inorganic ion concentrations in Uronema filificum

Preliminary results on the inorganic ion content of a free-living, marine ciliate, *Uronema filificum,* were reported earlier (Kehlenbeck *et al.,* 1965). For comparative purposes more complete data are shown in Table II. As in the case of *M. avidus, U. filificum* had a greater $[K^+]_i$ than $[K^+]_o$ in 50% and in 100% sea water while $[Na^+]_i$ and $[Cl^-]_i$ were lower than $[Na^+]_o$ and $[Cl^-]_o$. *U. filificum* had a greater $[K^+]_i$ and a lower $[Cl^-]_i$ than *M. avidus.*

TABLE III

Intracellular inorganic ion concentrations of M. avidus *equilibrated for 30 min in various test solutions*

	mM/kg cells		
	[±2–6 mM (SE) in 3–6 determinations]		
	$[Na^+]_i$	$[K^+]_i$	$[Cl^-]_i$
100% Sea water	92.3	78.3	91.5
50% Sea water	48.6	61.5	31.9
50% Sea water 0.72 *M* Sucrose	85.5	56.7	93.5
100% Sea water 50% Na^+	53.6	82.1	98.9
100% Sea water 50% Cl^-	108.7	81.7	60.1

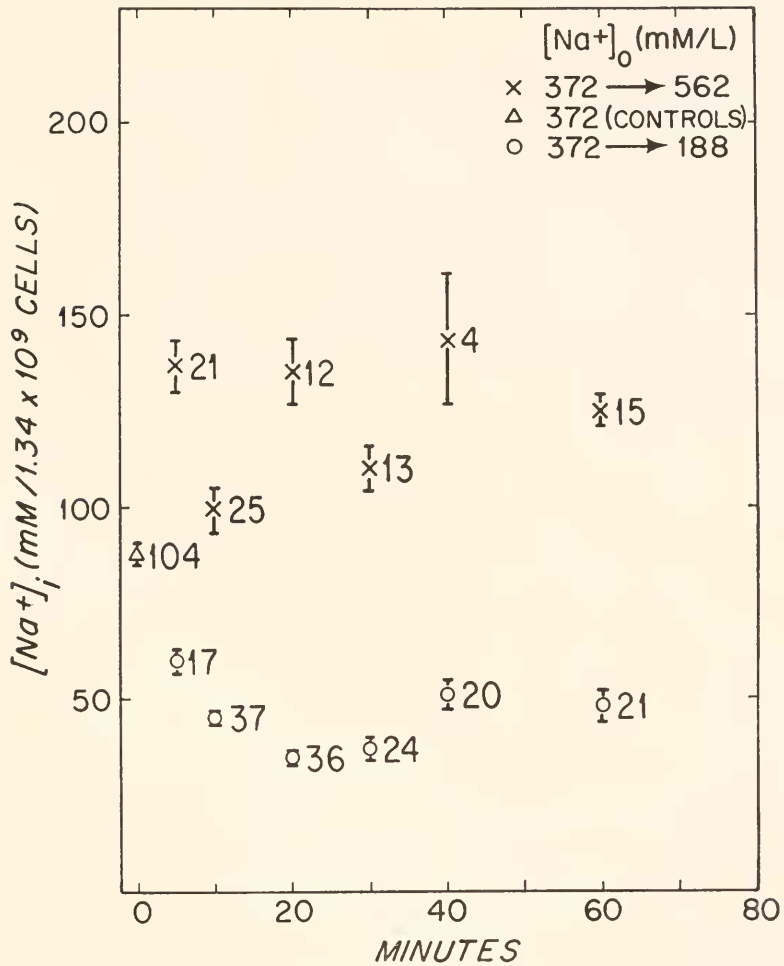

FIGURE 5. Net influx and net efflux of Na^+ in *M. avidus*.

Ion substitution experiments

The effects of ion substitutions on intracellular inorganic ion concentrations were analyzed 30 min after *M. avidus,* subcultured in 100% sea water-culture medium, was equilibrated in test solutions (Table III). When 50% of the Na^+ in the final test solution was replaced with choline$^+$, $[Na^+]_i$ decreased ($p < 0.001$); however, $[K^+]_i$ was unaffected by a 50% reduction in $[K^+]_o$ replaced by choline$^+$. $[Cl^-]_i$ was reduced when propionate$^-$ and SO_4^{--} replaced 50% of the Cl^- in the external solution ($p < 0.001$). $[Ca^{++}]_i$ and $[Mg^{++}]_i$ were not altered by any of the external changes in ion composition or ion concentration tested. When sucrose isosmotic to sea water was substituted for 50% of the sea water, the intracellular concentrations of all ions except K^+ were maintained at the level found in cells in 100% sea water. $[K^+]_i$ was reduced ($p < 0.001$).

Contractile vacuole output

In *M. avidus* the fraction of cell volume eliminated by the contractile vacuole per unit time increased with decreasing external osmotic pressure (Fig. 8). On transfer to media of osmolarity greater than sea water, there was a slight decrease in the rate of fluid output by the vacuole. In 25% sea water the contractile vacuole expelled an amount of fluid equal to the cell volume in about a half-hour; in 175% sea water in about 2 hours. That the contractile vacuole output rate was responsive to changes in external osmolarity and not ionic strength was shown when the external salt concentration was decreased by 50% but the osmotic pressure was maintained at the value for 100% sea water by the addition of sucrose. In this experiment the average fraction of cell volume turnover/min was 0.011 ($\pm$ 0.003, SE; 11 determinations). The output rates of ciliates in 50% sea water and in the sucrose-sea water solution were significantly different ($p < 0.05$).

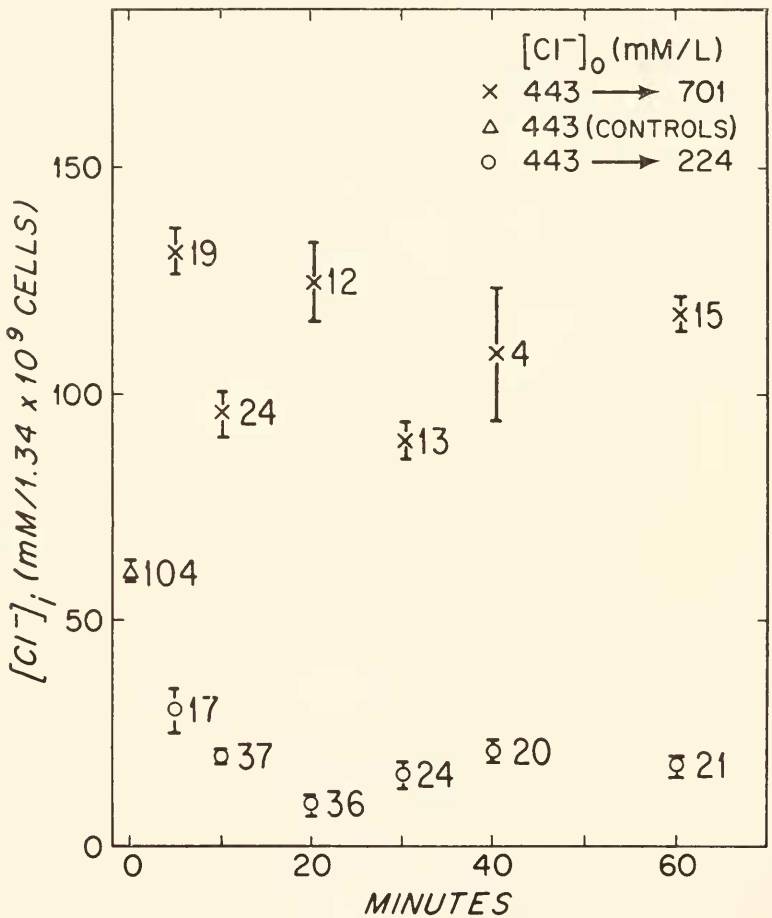

FIGURE 6. Net influx and net efflux of Cl^- in *M. avidus*.

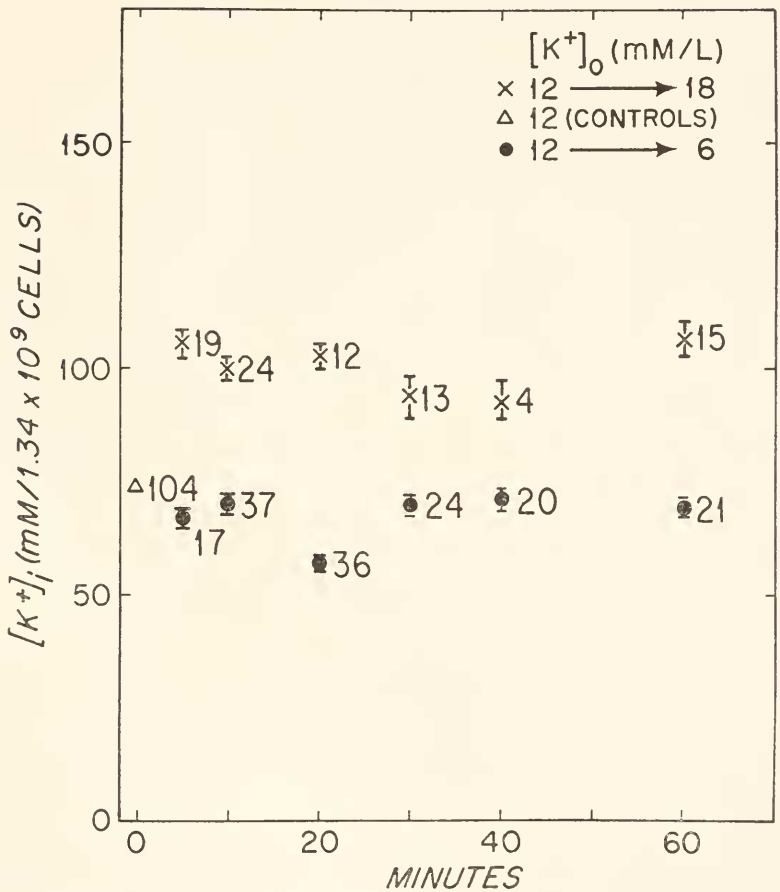

FIGURE 7. Net influx and net efflux of K^+ in *M. avidus*.

Cell volume

When exposed to environments more dilute or more concentrated than sea water, *M. avidus* swelled or shrank, respectively. Figure 9 illustrates the volumes of ciliates equilibrated for 30 min in media of different salinities. After being in the new environment for about 90 min, the ciliates almost regained their original volumes (Fig. 10).

DISCUSSION

For both *M. avidus* and *U. filificum* the concentration gradients of K^+, Na^+ and Cl^- between the ciliates and the environment were typical for animal cells. The mechanisms responsible for the maintenance of the gradients are not known. If the cytoplasm is electrically negative to the external medium, the Na^+ gradient is far from thermodynamic equilibrium. Therefore active transport of Na^+ is likely since the ciliates are permeable to Na^+. The contractile vacuole may be responsible for

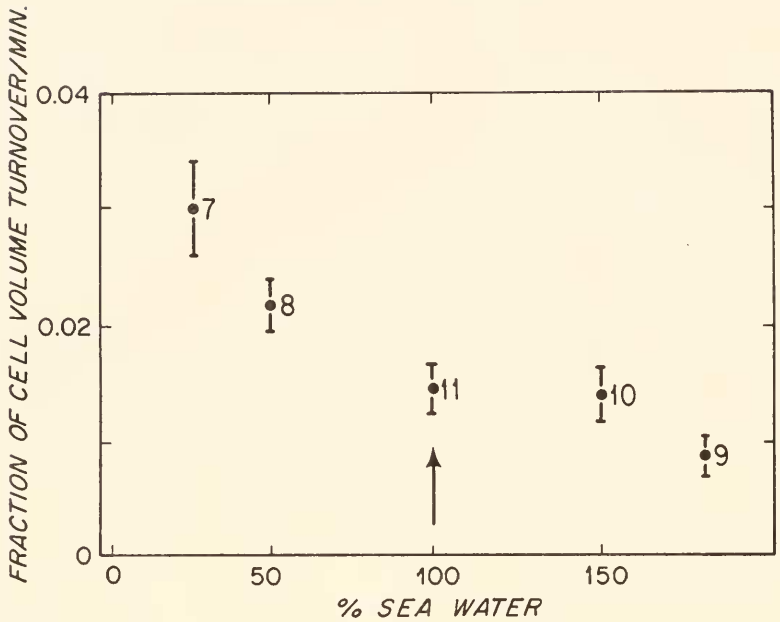

FIGURE 8. Vacuolar output of *M. avidus* (expressed as fraction of cell volume turnover/min) as a function of sea water concentration.

active extrusion of Na^+ (Chapman-Andresen and Dick, 1962; Marshall, 1966; Dunham and Stoner, 1967). Maintenance of $[K^+]_i$ may be passive or may depend on active accumulation as in some fresh water protozoa (Dunham and Child, 1961; Conner, 1967).

M. avidus placed in 50% sea water with sufficient sucrose to make it isosmotic to 100% sea water had $[Na^+]_i$ and $[Cl^-]_i$ at the levels found in ciliates in 100% sea water, *i.e.*, significantly higher than in ciliates in 50% sea water. $[Na^+]_i$ and $[Cl^-]_i$ are, therefore, functions of the external osmolarity and not of ionic strength. However, the decrease in $[Na^+]_i$ in response to propionate$^-$ and SO_4^{--} substitutions in the medium are not easily reconciled with the maintenance of normal $[Na^+]_i$ and $[Cl^-]_i$ in 50% sea water + sucrose.

The relationship between the rate of fluid output by the contractile vacuole and the osmolarity of the external medium suggests an osmoregulatory function for the contractile vacuole. Kitching (1936) also observed an increased rate of contractile vacuole output in marine peritrich ciliates upon transfer to dilute sea water but did not measure output rates in sea water concentrations above 100%. The continued function of the vacuole of *M. avidus* in all sea water concentrations tested, including 200%, suggests that the cells are hyperosmotic to all of the media. Since vacuolar output was only slightly reduced after transfer to media more concentrated than 100% sea water, the intracellular osmolarity must increase after the transfer, such that the difference between intracellular and external osmolarities does not change much. On the other hand, the contractile vacuole may be removing water from a source other than osmotic entry, such as water derived from metabolism.

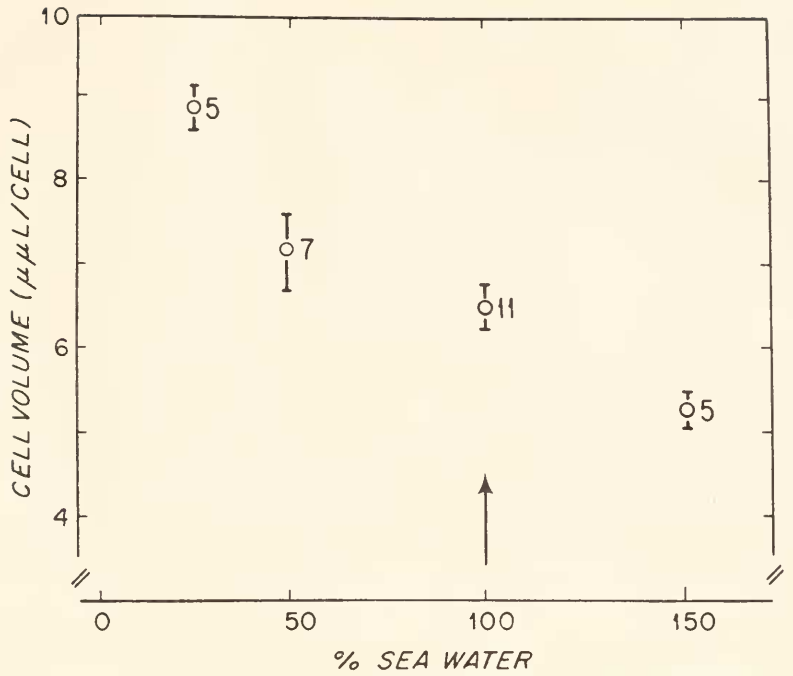

FIGURE 9. Changes in cell volume of *M. avidus* equilibrated for 30 min in different concentrations of sea water.

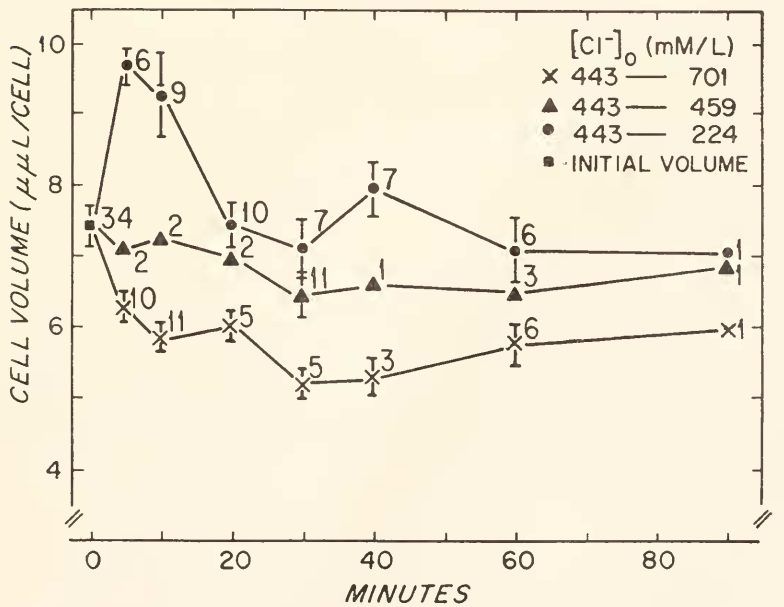

FIGURE 10. Cell volumes of *M. avidus* at different times after transfer to different salinities.

In fresh-water protozoa, metabolic water is a minor fraction of vacuolar output (Kitching, 1967); however, metabolic water might be a significant aspect of water balance in marine protozoa.

After osmotic challenges, cell volume changed passively, but then returned toward the volume of ciliates kept in 100% sea water (Fig. 10). In no way can the volume recovery be accounted for as a purely passive process. This restoration of volume was particularly evident with ciliates transferred to 50% sea water. This aspect of volume regulation has been observed in few other protozoans (Kamada, 1935; Mast and Hopkins, 1941; Dunham and Stoner, 1967); there is no clear case of volume restoration in a marine ciliate (Kitching, 1967). Restoration of cell volume might be accomplished by maintenance of a rate of vacuolar output different from the rate of passive entry of water until volume was restored, after which time vacuolar output would be kept equal to passive entry in order to maintain constant cell volume. Redistribution of solutes, particularly Na^+ and Cl^-, after osmotic challenges must also play a role in regulation of cell volume.

The time required for the contractile vacuole to discharge a volume of fluid equal to the cell volume was 1.1 hour in 100% sea water. In fresh-water protozoans, turnover of cell water is much more rapid (4–45 min; Kitching, 1938). All marine ciliates have slower rates than fresh-water ciliates. Kitching (1938) has reported values of 2¾–4¾ hours for marine peritrichs. *M. avidus,* however, is smaller than the marine ciliates used in previous studies. With its larger surface/volume ratio, a higher rate of water entry per unit volume is expected.

Summary

1. The euryhaline marine ciliate, *Miamiensis avidus,* was investigated for its ability to regulate solutes and water when exposed to different external salinities.

2. In 100% sea water-culture medium, *M. avidus* had the following inorganic ion concentrations (m*M*/kg cells): Na^+—87.9; K^+—73.7; Ca^{++}—3.7; Mg^{++}—28.5; Cl^-—60.8.

3. In 100% sea water-culture medium, $[Na^+]_i$, $[Cl^-]_i$, $[Mg^{++}]_i$ and $[Ca^{++}]_i$ were lower than the environmental values and $[K^+]_i$ was greater than $[K^+]_o$. $[Na^+]_i$ and $[Cl^-]_i$ changed with changes in external salinity, but were kept lower than $[Na^+]_o$ and $[Cl^-]_o$. $[K^+]_i$, $[Mg^{++}]_i$ and $[Ca^{++}]_i$ were maintained at fairly constant internal concentrations.

4. The contractile vacuole output was related to external osmolarity. Osmolarities greater than that of 100% sea water resulted in decreased vacuole output. In dilute sea water, output increased.

5. Cell volume determinations indicated a return toward the original volume after swelling or shrinking caused by transfer to media of different osmolarities.

6. The results suggest that *M. avidus* maintains itself hyperosmotic to the environment at all salinities. The contractile vacuole regulates cell volume by expelling water that enters passively.

LITERATURE CITED

Chapman-Andresen, C., and D. A. T. Dick, 1962. Sodium and bromine fluxes in the amoeba *Chaos chaos* L. *C. R. Trav. Lab. Carlsberg,* 32: 445–469.

Conner, R. L., 1967. Transport phenomena in Protozoa. *In:* Chemical Zoology, vol. I, Protozoa. Pp. 309–350. G. W. Kidder (Ed.), Academic Press, New York.

Dunham, P. B., and F. M. Child, 1961. Ionic regulation in *Tetrahymena*. *Biol. Bull.*, **126**: 373–390.

Dunham, P. B., and L. C. Stoner, 1967. Regulation of intracellular sodium concentration by the contractile vacuole in *Tetrahymena*. (abstr.) *J. Protozool.* (Suppl.), **14**: 34.

Kamada, T., 1935. Contractile vacuole of *Paramecium*. *J. Fac. Sci., Univ. Tokyo,* **4**: 49–62.

Kehlenbeck, E. K., P. B. Dunham and G. G. Holz, Jr., 1965. Inorganic ion concentrations in a marine ciliate, *Uronema*. (abstr.) *Progr. Protozool.* (London), Excerpta Medica, Amsterdam.

Kitching, J. A., 1936. The physiology of contractile vacuoles. II. The control of body volume in marine Peritricha. *J. Exp. Biol.*, **13**: 11–27.

Kitching, J. A., 1938. Contractile vacuoles. *Biol. Rev.*, **13**: 403–444.

Kitching, J. A., 1967. Contractile vacuoles, ionic regulations, and excretion. *In:* Research in Protozoology, vol. 1. Pp. 307–336. E. Chen (Ed.), Pergamon Press, New York.

Marine Biological Laboratory Formulae and Methods V, 1964. Pp. 55. G. M. Cavanaugh (Ed.), M.B.L., Woods Hole, Mass.

Marshall, J. M., 1966. Intracellular transport in the amoeba *Chaos chaos*. Symp. Internat. Soc. Cell Biol. V. Intracellular Transport, Academic Press, New York.

Mast, S. O., and D. L. Hopkins, 1941. Regulation of water content of *Amoeba mira* and adaptation to changes in the osmotic concentration of the surrounding medium. *J. Cell. Comp. Physiol.*, **17**: 31–48.

Moewus, L., 1963. Studies on a marine parasitic ciliate as a potential virus vector. *In:* Symp. on Marine Microbiology. Pp. 366–379. C. H. Oppenheimer (Ed.), Charles C. Thomas, Springfield, Ill.

Thompson, J. C., and L. Moewus, 1964. *Miamiensis avidus* n.g., n.sp., a marine facultative parasite in the ciliate order Hymenostomatida. *J. Protozool.*, **11**: 378–381.

Thompson, J. C., and E. S. Kaneshiro, 1968. Redescription of *Uronema filificum* and *U. elegans*. *J. Protozool.*, **15**: 141–144.